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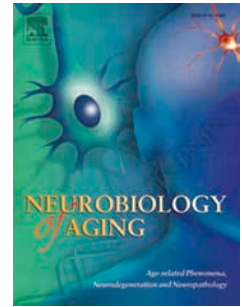
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Assessment of ZnT3 and PSD95 protein levels in Lewy body dementias and Alzheimer's disease: association with cognitive impairment.

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Key words: Alzheimer's disease, Dementia with Lewy bodies, Parkinson's disease with dementia, synaptic dysfunction, zinc, beta amyloid, tau, cognitive impairment

Abstract

The loss of zinc transporter 3 (ZnT3) has been implicated in age-related cognitive decline in mice and the protein has been associated with plaques. We investigated the levels of ZnT3 and PSD95, a marker of the post-synaptic terminal, in people with Parkinson's disease dementia (PDD n=31), dementia with Lewy bodies (DLB n=44), Alzheimer's disease (AD n=16) and controls (n=24), using semi-quantitative western blotting and immunohistochemistry in three cortical regions. Standardized cognitive assessments during life and semi-quantitative scoring of A β , tau and α -synuclein at post-mortem were used to investigate the relationship between ZnT3 and PSD95, cognition and pathology.

Associations were observed between ZnT3 and PSD95 levels in prefrontal cortex and cognitive impairment ($p=0.001$ and $p=0.002$ respectively), and between ZnT3 levels in the parietal cortex and cognitive impairment ($p=0.036$). Associations were also seen between ZnT3 levels in cingulate cortex and severity of amyloid- β ($p=0.003$) and tau pathology ($p=0.011$). DLB and PDD were characterized by significant reductions of PSD95 ($p<0.05$) and ZnT3 ($p<0.001$) in prefrontal cortex compared to controls and AD. PSD95 levels in the

parietal cortex were found to be decreased in AD cases compared to controls ($p=0.02$) and PDD ($p=0.005$).

This study has identified Zn^{2+} modulation as a possible novel target for the treatment of cognitive impairment in DLB and PDD, and the potential for synaptic proteins to be utilised as biomarkers for the differentiation of DLB and PDD from AD.

Introduction

The Lewy body dementias, incorporating dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) are collectively the second most common neurodegenerative dementia (Aarsland, et al., 2008) and are pathologically characterized by α -synuclein, with varying amounts of A β and tau aggregates in addition to synaptic loss (Dickson, 2002). Clinical hallmarks include fluctuating and deteriorating cognition, hallucinations, and parkinsonism (McKeith, et al., 2005).

There is an increasing recognition of the importance of metal ions, in particular zinc, in neurodegenerative disorders (Sensi, et al., 2009). Zn^{2+} is sequestered into synaptic vesicles exclusively by the synapse-specific member of the zinc transporter family, ZnT3, prior to release with neurotransmitter as a modulator of neural transmission (Lee, et al., 2011, Palmiter, et al., 1996, Sensi, et al., 2009); thus ZnT3 is vital for correct synaptic release and regulation of Zn^{2+} .

Ablation of the ZnT3 gene in mice (ZnT3 KO) resulted in age-related memory impairment (Adlard, et al., 2010, Sindreu, et al., 2011). It was suggested that ZnT3 mediated synaptic Zn^{2+} homeostasis is important for maintenance of cognition and that loss of ZnT3 and the consequent dyshomeostasis of synaptic Zn^{2+} may adversely affect memory and cognition. ZnT3 KO mice also showed decreases in PSD95 (Adlard, et al., 2010). ZnT3 has been found associated with plaques in AD (Zhang, et al., 2008). Furthermore, Zn^{2+} has been shown to facilitate aggregation of A β (Bush, et al., 1994, Curtain, et al., 2001), tau (Boom, et al., 2009, Mo, et al., 2009) and α -synuclein (Paik, et al., 1999, Yamin, et al., 2003).

PSD95 is a post synaptic protein key to the cellular scaffolding and regulation of membrane expressed neurotransmitter receptors at the post synaptic terminal. PSD95 is widely recognised to have roles in synaptic function including regulation of the localisation of membrane proteins and protein trafficking (Han and Kim, 2008). Reductions in PSD95 have been observed in AD (Gylys, et al., 2004, Love, et al., 2006), although not consistently

(Leuba, et al., 2008), and implicated in learning and memory deficits in mice (Migaud, et al., 1998, Nyffeler, et al., 2007).

Despite the possible roles of Zn^{2+} and synaptic dysfunction in cognitive, behavioural and pathological aspects of DLB, PDD and AD, the status of ZnT3 and PSD95 in DLB or PDD has not been investigated. Hence, the present study examined the concentration and distribution of ZnT3 and PSD95 in post mortem brain tissue retrieved from a large cohort of DLB, PDD and AD patients, well characterized clinically and neuropathologically, and controls. We aimed to determine what the relationships were between these proteins and cognition, and the neuropathological hallmark lesions $A\beta$, tau and α -synuclein in DLB, PDD and AD. It was hypothesised, on the basis of previous literature, that reduced ZnT3 and PSD95 would be associated with cognitive impairment in DLB and PDD, specifically in the prefrontal cortex as this region is selectively involved in the most prominent cognitive changes in these dementias. Furthermore, it was hypothesised that AD cases would have a stronger association to changes in the parietal cortex, again due to the prominence of atrophy and degenerative changes this region in AD (Guadagna, et al., 2012, Jacobs, et al., 2012, Jacobs, et al., 2011, Kirvell, et al., 2006).

2. Methods and Materials

2.1 Participants, diagnosis and assessment

Post-mortem brain tissue was obtained from several sources; University Hospital Stavanger (Norway), the MRC London Neurodegenerative Diseases Brain Bank, the Thomas Willis Oxford Brain Collection and the Newcastle Brain Tissue Resource. The UK brain banks are part of the Brains for Dementia Research Network. All participants gave informed consent for their tissue to be used in research and the study had ethics approval from the National Research Ethics Service (08/H1010/4). Table 1 shows the demographic details of the patients and controls. Biochemical and histopathological analysis was undertaken on prefrontal cortex (Brodmann area, BA, 9), anterior cingulate gyrus (BA24) and parietal cortex (BA40). BA9 was selected due to its proposed role in executive function and cognition (Fuster, 2001),

decline of which is a cardinal symptom of DLB and PDD, BA24 was selected for the early development of pathology encountered in this region in DLB and PDD (Alafuzoff, et al., 2009) whilst BA40 was selected because of its pathological predominance in AD as opposed to DLB and PDD (Alafuzoff, et al., 2008, Guadagna, et al., 2012, Jacobs, et al., 2012, Jacobs, et al., 2011, Kirvell, et al., 2006).

Neuropathological assessment was performed according to standardised neuropathological scoring/ grading systems, including Braak staging, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scores, Newcastle/ McKeith Criteria for Lewy body disease, National Institute on Aging - Alzheimer's Association (NIA-AA) guidelines and phases of amyloid- β (A β) deposition (A β -phases) (Braak, et al., 2006, McKeith, et al., 2005, Mirra, et al., 1991, Montine, et al., 2012, Thal, et al., 2002). Controls were neurologically normal, with only mild age associated neuropathological changes (e.g., neurofibrillary tangle Braak stage \leq II) and no history of neurological or psychiatric disease.

Cognitive impairment data consisted of the last Mini-Mental State Examination (MMSE) scores a maximum of two years prior to death (Folstein, et al., 1975). Although the MMSE is not the most sensitive tool for some aspects of DLB and PDD such as executive function (McKeith, et al., 2005), its ubiquity made it the only feasible cognitive measure for this study. Patients and controls were categorised according to cognitive impairment in the following manner; 'unimpaired cognition' – for individuals classified by the brain bank(s) as being clinical controls, 'neurodegenerative disease without dementia' – for individuals with MMSE scores of 25 to 30 and no antemortem diagnosis of dementia, 'mild dementia' – for individuals with MMSE scores from 17 to 24, 'moderate dementia' – for individuals with MMSE scores of 10 to 16, 'severe dementia' for individuals with MMSE scores of 9 or less. Final diagnoses for patients are clinico-pathological consensus diagnoses incorporating the one-year rule to differentiate DLB and PDD (McKeith, et al., 2005). Earlier reports of at least some of the cohorts showed that there was a very good match between clinical and pathological diagnosis (Aarsland, et al., 2005).

2.2 Immunohistochemistry

Sections (7µm) of paraffin embedded tissue from BA9 were cut and de-waxed using xylene and alcohol. Epitope retrieval consisted of microwaving the sections in citrate buffer (Yamashita, 2007). The primary antibodies were anti-ZnT3 (Synaptic Systems, 197-002, rabbit polyclonal, 1:200) and anti-PSD95 (Abcam ab18258, rabbit polyclonal, 1:400). Di-amino-benzidine (DAB, sigma) enhanced with nickel was the visualisation reagent and a haematoxylin counter-stain was employed.

Semi-quantitative assessments of Aβ, tau and α-synuclein pathology were conducted blind to clinical diagnosis, by neuropathologists (JA & TH), using a scale of 0 (none), 1 (sparse), 2 (mild) and 3 (severe/frequent) to score sections from BA9, BA24 and BA40. For detection of senile Aβ plaques sections were stained with an anti-Aβ 1E8 or 4G8 antibody (17-24, mouse monoclonal, Covance, SIG-39220) at 1:1000. Tau immunohistochemistry (AT8 mouse monoclonal antibody, 90206, Innogenetics, at 1:200) and silver impregnation (Gallyas or modified Bielschowsky) were used to detect neurofibrillary tangles, neuritic plaques, dystrophic neurites and neuropil threads. α-synuclein pathology was detected using NCL-SYN antibody (KM51, Novocastra Laboratories, Leica Biosystems) at 1:200. Examples of these three stains and each category of semi-quantitative score can be seen in figure 5.

2.3 Preparation of tissue samples for western blotting

Preparation of tissue for western blotting was as previously described (Kirvell, et al., 2006). Briefly, 500mg of frozen tissue was taken from each brain region. Meninges, white matter, blood vessels and clots were dissected from the frozen tissue to leave approximately 200mg of grey matter which was homogenised in ice cold buffer containing 50mM tris-HCL, 5mM EGTA, 10mM EDTA, 'complete protease inhibitor cocktail tablets' (Roche, 1 tablet per 50ml of buffer), and 2µg/ml pepstatin A dissolved in ethanol:DMSO 2:1 (Sigma). Buffer was used at a ratio of 2ml to every 100mg of tissue and homogenisation performed using an IKA Ultra-Turrax mechanical probe (KIA Werke, Germany) until the liquid appeared homogenous.

Protein concentration was established using the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific), briefly 10µl of crude homogenate was diluted 1:50 and read in triplicate at 595nm using a FlexStation 3 (Molecular Devices). Concentration was calculated using a BSA standard curve run at the same time as samples.

2.4 Western Blotting

Crude brain homogenate was diluted 4:5 with 5x sample buffer (Genscript MB01015), boiled for 5 minutes then stored at -20°C. Samples were loaded at 20µg/ml total protein on 10% SDS-polyacrylamide gel for protein separation, transferred to nitrocellulose membrane (Hydrobond-C, Amersham) and probed with either anti-PSD-95 (Thermo Scientific, MA1-045, clone 6G6-1C9, mouse monoclonal IgG2a, 1:7000) or anti-ZnT3 (Synaptic Systems, 197-002, rabbit polyclonal, 1:5000) and the relevant secondary antibody (IRDye from LI-COR). Bands were detected using an Odyssey infrared fluorescent scanner, the integral of intensity quantified using Odyssey infrared imaging systems application software version 3.0.16 and expressed as ratios to rat cortex in arbitrary units.

2.5 Statistical preparation and analysis

The normality of the data for each protein was determined using the Shapiro-Wilk test in SPSS and normalised where necessary. Regression analysis (enter method in SPSS) of normalised data was used to determine the effect of confounding variables (see table 1) on protein values; gender significantly predicted ZnT3 values and post-mortem delay (PMD) significantly predicted PSD95 values for BA9, ZnT3 values for BA24 were significantly predicted by age at death and pH (PSD95 values had no relationship to any confounding variables), whilst PMD was a significant predictor of both protein values in BA40. In each case, the protein values were subsequently expressed as residuals (unstandardised) created from the regression analysis, to eliminate the confounding effect of the demographic variables on the protein values. Unstandardised residuals were used in all subsequent analyses. We tested for differences in protein levels between groups using one-way ANOVA and Bonferroni post-hoc test in SPSS.

3. Results

AD patients were significantly older at death than controls ($p=0.007$) or patients with DLB ($p=0.011$) or PDD ($p=0.003$). There were no significant differences in PMD, tissue pH or gender between diagnostic groups (table 1).

3.1 Differences in the levels of ZnT3 and PSD95 between diagnostic groups.

The image in figure 1 illustrates the typical western blot of ZnT3 and PSD95, showing diagnostic and brain regional specific reductions and confirming the presence of a single band at approximately 40kDa for ZnT3 and 97kDa for PSD95.

Significant differences ($p<0.05$) were detected (using semi-quantitative western blotting) in the levels of ZnT3 and PSD95 in the prefrontal cortex (BA9) according to diagnosis (Figure 1A and 1B). Significant reductions in ZnT3 were seen in both PDD (28%, $p=0.0004$) and DLB (19%, $p=0.001$) compared to controls (details of statistical analysis are provided in the figure caption). There was no significant alteration in the level of ZnT3 protein in patients with AD compared to any other group. With respect to PSD95, mean levels were lower in both PDD (28% $p=0.0001$) and DLB (17% $p=0.001$) compared to controls. Furthermore, mean PSD95 levels were lower in PDD compared to AD (19% $p=0.012$) and DLB (13% $p=0.036$). In support of the changes seen by western blotting, figure 2 illustrates typical immuno-labelling of prefrontal cortex sections from each diagnostic group for ZnT3 (figure 2A-D) and PSD95 (figure 2F-I). In both cases PDD sections have a considerably lower staining intensity than control and AD, supporting the evidence from western blotting in figure 1A and 1B. The staining intensity for either protein did not differ greatly between DLB, AD or controls.

There was no significant difference in the levels of ZnT3 in the cingulate gyrus (BA24) between the diagnostic groups (figure 1C, $p>0.05$). However, mean PSD95 levels in patients with AD were significantly elevated by 23% compared to PDD patients ($p=0.027$) and by 25% compared to control (figure 1D $p=0.037$).

There was no significant difference in the levels of ZnT3 in the parietal cortex (BA40) between the diagnostic groups (figure 1E, $p>0.05$). However PSD95 levels were reduced in people with AD by 22% compared to controls ($p=0.02$) and by 24% compared to people with PDD (figure 1F $p=0.005$).

3.2 Reductions in ZnT3 and PSD95 were associated with cognitive impairment.

The reduced levels of ZnT3 and PSD95 detected in BA9 were found to be associated with cognitive impairment (figure 3A and 3B; $R^2=0.297$, $\beta=-0.339$, $df\ 2,77$, $t=-3.325$, $p=0.001$ and $\beta=-0.325$, $df\ 2,77$, $t=-3.194$, $p=0.002$ respectively), this analysis included all control, DLB and PDD subjects. In addition, in pairwise comparisons, patients with mild dementia had mean ZnT3 and PSD95 levels that were significantly lower than individuals with unimpaired cognition (ZnT3, 24% $p=0.013$, PSD95 19% $p=0.028$) and levels of both proteins were significantly lower in people with moderate dementia (both 24%, ZnT3 $p=0.001$, PSD95 $p=0.0001$) and with severe dementia (both 24%, ZnT3 $p=0.005$, PSD95 $p=0.002$) compared to controls. Upon inclusion of AD patients in the analysis, ZnT3 and PSD95 levels in BA9 were still associated with cognitive impairment but the association was weaker (ANOVA, $F=14.282$, $p<0.001$ $R^2=0.243$, $\beta=-0.320$, $df\ 2,89$, $t=-3.271$, $p=0.002$ and $\beta=-0.283$, $df\ 2,89$, $t=-2.895$, $p=0.005$ respectively).

Reduced levels of ZnT3 in BA40 were also associated with cognitive impairment (figure 3C, ANOVA, $F=4.540$, $R^2=0.48$, $\beta=-0.218$, $df\ 1,91$, $t=-2.131$, $p=0.036$) according to regression analysis of all controls and dementia patients, but were not significantly different between people with different severities of cognitive impairment (one-way ANOVA $p>0.05$). When AD patients were excluded there was no significant relationship between cognition and either protein in BA40 ($p>0.05$). No associations were found between ZnT3 or PSD95 levels and cognition in BA24 ($p>0.05$, data not shown).

3.3 Relationships between synaptic proteins and neuropathological scores

In BA24, ZnT3 levels were inversely associated with the semi-quantitative scores for both A β pathology (figure 4A, ANOVA, $F=9.292$, $R^2=0.91$, $\beta=-0.301$, $df\ 1,93$, $t=-3.048$, $p=0.003$)

and tau pathology (figure 4B, ANOVA, $F=6.687$, $R^2=0.065$, $\beta=-0.255$, $df\ 1,96$, $t=-2.586$, $p=0.011$) according to regression analysis of all dementia patients and controls. However there was no association between ZnT3 and these scores for other brain regions ($p>0.05$, data not shown). Furthermore, there was no association between ZnT3 levels and α -synuclein pathology in any of the brain regions examined ($p>0.05$, data not shown). PSD95 did not relate to any neuropathological scores in any brain region ($p>0.05$, data not shown).

4. Discussion

The principal observation of this study was an association between reduced ZnT3 and PSD95 in the prefrontal cortex and cognitive impairment in DLB and PDD cases, which is consistent with the selective involvement of this region in Lewy body dementias. Other important findings were: decreased levels of ZnT3 and PSD95 in the prefrontal cortex of PDD and DLB cases compared to controls; increased PSD95 in the cingulate gyrus of AD cases compared to controls and PDD; and decreased PSD95 levels in the parietal cortex of AD cases compared to controls and PDD. Additionally, levels of ZnT3 inversely correlated with both $A\beta$ and tau pathology in the cingulate gyrus (BA24) but not in the other regions studied.

Significant strengths of this study include the large number of patients with DLB and PDD and the undertaking of brain region specific pathology scores. Additionally, the semi-quantitative changes observed by western blotting were supported by a small selection of cases examined immunohistochemically, although it is recognised that the latter technique was not quantified. One limitation inherent in post-mortem studies is the differences in demographics and epiphenomena between groups, this has been addressed statistically through the creation of residuals. Another limitation is the observational nature of the results reported, although this is mitigated by the previously reported mechanistic animal studies on ZnT3 implicating this protein in cognition (Adlard, et al., 2010).

The observed reductions in ZnT3 and PSD95 in prefrontal cortex of DLB and PDD patients are consistent with consensus that these dementias are characterized by greater frontal

degeneration in comparison to AD, whilst the greater reduction in these proteins in BA40 in AD is consistent with more extensive parietal degeneration in AD (Burton, et al., 2012).

Increases in PSD95 have been previously reported in AD cases, although this was in frontal cortex (BA9), and was attributed to compensatory mechanisms at the post-synaptic terminal (Leuba, et al., 2008).

It has been previously reported that ablation of ZnT3 by gene deletion in mice is associated with age-related cognitive deficits (Adlard, et al., 2010). One possible explanation for this is that Zn^{2+} is co-released with glutamate, acting as a long term modulator of synaptic plasticity, and thus is important for cognition (Sensi, et al., 2009). Our findings suggest this association between a loss of ZnT3 and cognitive deficits extends to humans and is more pronounced in DLB and PDD than AD. Controls, DLB and PDD patients were included in the analysis to achieve a gradation of all stages of cognitive impairment. This association is probably related to frontal synaptic dysfunction, and as reduced PSD95 was also predictive of cognitive deficits. Interestingly, decreased Zn^{2+} concentration at the post-synaptic density has been shown *in vitro* to cause reductions in PSD95 (Grabrucker, et al., 2011a).

Furthermore, Zn^{2+} has been implicated in A β and tau aggregation *in vitro* (Boom, et al., 2009, Bush, et al., 1994, Curtain, et al., 2001, Mo, et al., 2009). In this context our findings of an association between reduced ZnT3 in BA24 and increases in both A β and tau pathologies are consistent with the *in vitro* studies and suggest a loss of regulation of synaptic Zn^{2+} to have a contributory role in the production of pathology within this region. BA24 is noted for the early development of pathology in DLB and PDD (Alafuzoff, et al., 2009). Indeed, the present findings may support Adlard and colleagues who proposed a ‘dual hit’ model in which dyshomeostasis of synaptic Zn^{2+} denies zinc its role in cognition, as less Zn^{2+} is co-released with neurotransmitter, whilst allowing the build-up of Zn^{2+} destined to be packaged into vesicles to instead promote protein aggregation (Adlard, et al., 2010). A similar concept was proposed by Grabrucker and colleagues in response to their evidence of A β sequestration

of Zn^{2+} depleting the post-synaptic density (Grabrucker, et al., 2011b). The present evidence of associations between AD pathology and loss of Zn^{2+} regulation suggests these theories are relevant to the disease process in man.

If indeed some of the pathology is associated directly with zinc dyshomeostasis, there is no evidence from the present study that this is linked to cognitive impairment since the correlation between reductions in ZnT3 and cognitive impairment and between ZnT3 and AD pathology occurred in different brain regions. However, it should be noted that BA9 and BA24 are highly interconnected (Li, et al., 2013). Further mechanistic investigation, such as *in vitro* or animal studies, is warranted to elucidate the exact nature of the relationship between ZnT3, pathology and cognition. In particular it would be interesting to examine whether soluble oligomers of the key pathological proteins provide a better correlate to zinc dyshomeostasis in the other brain regions given the lack of relationship to pathological deposition outside of BA24.

To our knowledge this is the first study to connect ZnT3 to cognition in man despite investigations of ZnT3 levels in AD (although not DLB and PDD). Adlard and colleagues and Bjorklund and colleagues have reported reduced ZnT3 levels in AD patients. Whilst we did not find a significant reduction in ZnT3 levels in AD patients, ZnT3 levels were reduced compared to controls in BA9, BA24 and BA40 (by 16%, 11% and 18% respectively). This is despite a higher number of AD patients in the present study than that reported by Adlard and colleagues (Adlard, et al., 2010) and only 5 fewer AD patients than Bjorklund and colleagues (Bjorklund, et al., 2012) and serves to highlight the variability inherent in human post-mortem protein studies.

Our findings of reduced PSD95 and ZnT3 in prefrontal cortex of DLB and PDD but not AD or control suggest this could be explored as a biomarker for differentiating DLB and PDD from other aged individuals. There have been no reports concerning ZnT3 or PSD95 in CSF or blood; however some synaptic proteins can be detected in CSF, whilst others such as synaptophysin are not detectable (Davidsson, et al., 1999, Schlaf, et al., 1998).

Importantly, the modulation of Zn^{2+} by a compound (PBT2) is under investigation as a potential treatment for AD. PBT2 has been shown to improve cognition in AD patients possibly through reducing zinc mediated $\text{A}\beta$ aggregation and increasing intracellular bioavailability of Zn^{2+} , this latter action may offset the loss of ZnT3 (Adlard, et al., 2008, Crouch, et al., 2011, Lannfelt, et al., 2008). Our findings suggest a similar approach might be appropriate for DLB and PDD because of the possible contribution of Zn^{2+} dyshomeostasis (as a consequence of reduced ZnT3) to cognitive impairment in these dementias. Furthermore, approximately 90% of the DLB cases in this study had levels of AD pathology sufficient to meet the CERAD criteria for probable AD so it is conceivable that this concurrent pathology may be more susceptible to zinc modulation.

It is clear, both from our findings with PSD95 and from the general literature, that there are very likely multiple synaptic correlates of cognitive impairment, we have identified two. Thus, in conclusion, we present evidence that a loss of regulation of synaptic Zn^{2+} may have a role in cognitive impairment in the Lewy body dementias and raise the possibility of modulating Zn^{2+} as a disease-modifying approach in DLB and PDD.

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Potential Conflicts of Interest

Within the last three years, Prof. Clive Ballard has received research grants from Lundbeck pharmaceutical company and fees for consultancy or speaking from Lundbeck, Acadia, Bristol-Myer Squibb, Bial and Novartis pharmaceutical companies. Prof. Paul Francis reports grants from Alzheimer's Society during the conduct of the study and personal fees from Lundbeck and Linklaters LLP outside the submitted work. Prof. Aarsland received honoraria for presentations from Novartis, Lundbeck, and research support from GE Health. Prof. O'Brien reports personal fees from Bayer Healthcare, personal fees from GE Healthcare, personal fees from TauRx, grants, personal fees and other from Lilly, and personal fees from Cytos outside the submitted work. Tibor Hortobágyi has received salary support from the National Brain Research Programme (NAP), Hungary. The authors have no other potential conflicts of interest to report.

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Tables and Figures

Diagnosis	Gender (M/F) %	Age at death	PMD	pH	MMSE	Braak stage
Control (25)	60/40	79.8 \pm 1.5	39.1 \pm 4.6	6.47 \pm 0.05	n/a	1 (0-2)
PDD (34)	53/47	79.9 \pm 1.0	33.5 \pm 2.7	6.44 \pm 0.06	13 (0-27)	2 (0-6)
DLB (45)	58/42	81.3 \pm 1.0	42.4 \pm 4.2	6.34 \pm 0.06	13 (0-30)	4 (1-6)
AD (16)	31/69	88.0 \pm 2.0	34.9 \pm 6.0	6.30 \pm 0.08	10.5 (0- 19)	4 (4-6)

Table 1; Demographic characteristics of the subjects used in this study according to clinical diagnosis, (\pm SEM); age at death, PMD and pH are mean values, MMSE is the median score prior to death and Braak stage is median, both with range in brackets. One-way ANOVA and bonferroni post-hoc test revealed AD patients to have a significantly higher age at death than all other diagnostic groups (ANOVA; $F(3,106)5.015$, $p=0.003$, post-hoc; control $p=0.007$, PDD $p=0.003$ and DLB $p=0.011$). There was no significant difference between diagnostic groups for any of the other variables except cognition and pathology, for which subsequent analysis is shown in figures 3 and 4. PMD is post-mortem delay.

Figure 1: ZnT3 and PSD95 levels, from semi-quantitative western blotting in BA9, BA24 and BA40, according to diagnosis.

BA9

Statistical analysis was performed using One-way ANOVA and Bonferroni post-hoc test. In BA9: ZnT3 values (graph A) from controls were significantly higher than PDD ($p=0.0004$) and DLB ($p=0.001$), whilst PSD95 values (graph B) from controls were significantly higher than the PDD ($p=0.0001$) and DLB ($p=0.001$) groups. PSD95 values from the PDD were significantly lower than AD ($p=0.012$) and DLB ($p=0.036$). The ANOVA values were as follows; PSD95; $F=(3,108)12.809$, $p=0.0003$, ZnT3; $F=(3,111)9.409$, $p=0.0001$.

BA24

In BA24 there was no significant difference in ZnT3 levels between diagnostic groups (graph C, $p>0.05$) but PSD95 levels (graph D) were significantly higher in AD patients compared to controls ($p=0.037$) and PDD patients ($p=0.027$). ANOVA values: $F=(3,100)3.745$, $p=0.013$.

BA40

In BA40 there was no significant difference in ZnT3 levels between diagnostic groups (graph E). PSD95 values (graph F) in AD were significantly lower than control ($p=0.02$) and PDD ($p=0.005$). The ANOVA values were $F=(3,86)5.04$, $p=0.003$. The bars represent the mean values with SEM and n values in brackets under bars. For all figures $*=p<0.05$, $**=p<0.01$ and $***=p<0.001$.

The image (G) shows an example of the diagnosis specific reductions detected by western blot for PSD95 and ZnT3. PSD95 was visible on the red channel, ZnT3 on the green channel. The samples run on this gel were (from left to right); rat cortex,

BA9 AD (x3), BA24 AD (x2), BA40 AD (x5), mwm, rat cortex, BA40 AD (x1),

BA40 PDD (x9) and rat cortex.

Figure 2: Examples of ZnT3 and PSD95 immuno-labelling in prefrontal cortex according to diagnosis

Coronal sections of BA9 were labelled for ZnT3 (images A-F, A=control, B=AD, C=PDD, D=DLB, E=no primary antibody, F=control) or PSD95 (images G-K, G = control, H = DLB, I = PDD, J = AD, K = no primary antibody). It can be seen that PDD sections had very low levels of neuropil staining, almost comparable to no primary antibody, and that DLB cases had slightly lower staining than control and AD. Scale bars = 10 μ m and all images are 40X except image F which is 63X.

Figure 3: ZnT3 and PSD95 protein levels in BA9 and ZnT3 protein levels in BA40 predicted cognitive impairment.

BA9

Regression analysis showed ZnT3 and PSD95 levels in BA9 of control, DLB and PDD cases to be significant predictors of the cognitive impairment category ($R^2=0.297$, $\beta=-0.339$, $df\ 2,77$, $t=-3.325$, $p=0.001$ and $\beta=-0.325$, $df\ 2,77$, $t=-3.194$, $p=0.002$ respectively, ZnT3 graph A, PSD95 graph B). The ANOVA for the model was significant ($p=0.0001$). The cognitive impairment groups were; 'controls' (assigned a value of 1 and including all designated controls, ZnT3 $n=24$, PSD95 $n=23$), 'neurodegenerative disease without dementia' (cases assigned a value of 2 and based on a MMSE score of 25 to 30, ZnT3 $n=7$, PSD95 $n=8$), 'mild dementia' (assigned a value of 3 and based upon a MMSE score of 17 to 24, ZnT3 $n=12$, PSD95 $n=12$), 'moderate dementia' (assigned a value of 4 and based upon a MMSE score of

10 to 16, ZnT3 n=23, PSD95 n=22) and 'severe dementia' (assigned a value of 5 and based upon a MMSE score of 9 or less, ZnT3 n=19, PSD95 n=18). The difference in mean PSD95 and ZnT3 levels between cognitive impairment groups was analysed by one-way ANOVA and the Bonferroni post-hoc test, which revealed ZnT3 levels to be significantly higher in controls compared to people with severe dementia ($p=0.005$), moderate dementia ($p=0.001$) and mild dementia ($p=0.013$). PSD95 levels were calculated to be significantly higher in controls than in individuals with mild dementia ($p=0.028$), moderate dementia ($p=0.0001$) and severe dementia ($p=0.002$). The ANOVA values were $F(4.78)=6.819$, $p=0.00009$ for PSD95 and $F(4.80)=5.827$, $p=0.0004$ for ZnT3.

BA40

Additionally, regression analysis showed ZnT3 levels in BA40 to be a significant predictor of the cognitive impairment category ($R^2=0.048$, $\beta=-0.218$, $df\ 1,91$, $t=-2.131$, $p=0.036$ graph C). The ANOVA for the model was significant ($F=4.540$, $p=0.036$). The difference in mean ZnT3 levels between cognitive impairment groups was not found to be significant according to a Kruskal Wallis test ($p=0.093$). 'Unimpaired cognition' (1) n=22, 'neurodegenerative disease without dementia' (2) n=8, 'mild dementia' (3) n=14, 'moderate dementia' (4) n=24 and 'severe dementia' (5) n=25.

Figure 4: ZnT3 protein levels, from semi-quantitative western blotting, predicted the plaque and tangle scores in BA24.

Regression analysis showed ZnT3 values in BA24 to be a significant predictor of the semi-quantitative plaque score ($R^2=0.091$, $\beta=-0.301$, $df=1,93$, $t=-3.048$, $p=0.003$ graph A). The ANOVA for the model was significant ($F=9.292$, $p=0.003$). Plaque score; none (1) $n=40$, sparse (2) $n=27$, moderate (3) $n=16$ and severe (3) $n=12$. The difference in mean ZnT3 levels between plaque score groups was analysed by one-way ANOVA and the Bonferroni post-hoc test, which revealed ZnT3 levels to be significantly lower in subjects with a severe plaque score compared to subjects with a plaque score of absent ($p=0.029$); for the ANOVA $F(3,91)=3.331$, $p=0.023$.

Additionally, ZnT3 was found to be a significant predictor of the semi-quantitative tangle score in BA24 ($R^2=0.065$, $\beta=-0.255$, $df=1,96$, $t=-2.586$, $p=0.011$ graph B). The ANOVA for the model was significant ($F=6.687$, $p=0.011$). Tangle score; none (1) $n=46$, sparse (2) $n=31$, moderate (3) $n=12$ and severe (3) $n=9$. The difference in mean ZnT3 levels between tangle score groups was analysed by one-way ANOVA and the Bonferroni post-hoc test, which revealed no significant difference between tangle score groups.

Figure 5.1: Images of A β staining representative of the four categories of semi-quantitative score.

For each set of images, the whole section was viewed to determine the semi-quantitative score, representations of each score category are shown; and in each set image A is from a case given a score of absent/0, image B is from a case given a score of sparse/1, image C is from a case given a score of moderate/2 and image D is from a

case given a score of severe/3. The horizontal bar is 50 μ m. See the methods section for further details.

Figure 5.2; Images of tau staining representative of the four categories of semi-quantitative score.

Figure 5.3; Images of α -synuclein staining representative of the four categories of semi-quantitative score.

